Environmental Health Perspectives
Vol. 93, 105–109, 1991

Identification of Genes Associated with Tumor Suppression in Syrian Hamster Embryo Cells

by Roger W. Wiseman,* Jeffry C. Montgomery,* Junichi Hosoi,* Esther W. Hou,* Charles J. Cochran,* Patricia W. Lamb,* and J. Carl Barrett*

Loss of a tumor-suppressor gene function appears to play a critical role in the multistep process of neoplastic transformation of Syrian hamster embyro (SHE) cells *in vitro*. Clonal variants of two independent, preneoplastic cell lines have been isolated that have either retained (termed supB$^+$) or lost (termed supB$^-$) the ability to suppress the tumorigenicity of a highly malignant benzo[*a*]pyrene-transformed SHE cell line (BP6T) in cell hybrids. We have pursued several approaches in an attempt to identify genes that are responsible for tumor suppression in these cells. The only consistent differences detected in two-dimensional gel analyses of supB$^+$ and supB$^-$ cellular proteins were decreases in the levels of two high molecular weight isoforms of tropomyosin in supB$^-$ cells. Differential screening of a supB$^+$ cDNA library for genes that are preferentially expressed in supB$^+$ cells yielded cDNA clones for four genes, i.e., collagen type II, collagen type IX, H19, and a previously unidentified gene (clone 5). Nuclear run-on assays suggested that higher transcription rates were responsible for the increased steady-state levels of some of these transcripts in supB$^+$ cells. DNA sequence comparisons showed that two copies of a 9 bp element, previously identified in each of the mouse H19 enhancers, were also present in the 5′ flanking region of the rat type II collagen gene. A transcription factor that controls expression of the collagen and H19 genes through binding to this conserved motif would be an attractive candidate for the supB$^+$ gene or at least a mediator of the supB$^+$ phenotype.

Role of Tumor-Suppressor Genes in Multistep Carcinogenesis

It is now well established that the carcinogenic process requires the accumulation of multiple heritable alterations for the complete malignant transformation of normal cells (*1–3*). It is also clear that the inactivation of tumor-suppressor genes plays an important role in the genesis of a wide variety of human tumors (*3,4*). Strong evidence for this concept has been provided by studies of the suppression of tumorigenicity in somatic cell hybrids (*5,6*), genetic predispositions to cancer (*7,8*), and nonrandom chromosomal deletions or allelic losses in specific chromosomal regions (*9,10*). A number of these tumor-suppressor genes have recently been isolated by molecular cloning techniques (*11–15*), and in several cases tumor-suppressor activity has been demonstrated in functional assays (*16–18*).

*Laboratory of Molecular Carcinogenesis, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709.

Address reprint requests to R. W. Wiseman, Laboratory of Molecular Carcinogenesis, National Institute of Environmental Health Sciences, P. O. Box 12233, Research Triangle Park, NC 27709.

We have previously demonstrated that the loss or inactivation of a tumor-suppressor gene function is a critical event during the neoplastic transformation of carcinogen-treated or oncogene-transfected Syrian hamster embryo cells *in vitro* (*19,20*). The conversion of these normal cells to fully malignant tumor cells is clearly a multistep process (Fig. 1). Rare cells in carcinogen-treated cultures escape cellular senescence and form immortal (sen$^-$) cell lines (*21,22*). These cell lines

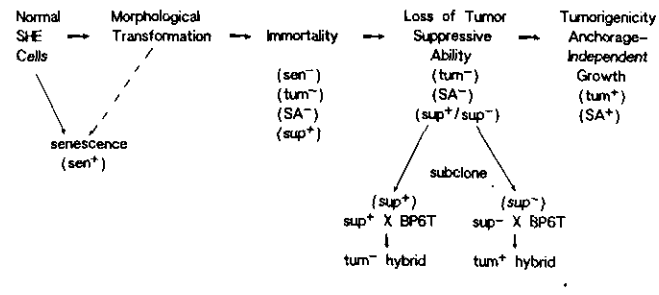

FIGURE 1. A pathway for neoplastic progression of Syrian hamster embryo cells *in vitro*.

are preneoplastic and require many population doublings *in vitro* prior to becoming anchorage independent (SA^+) or tumorigenic (tum^+) when injected subcutaneously into nude mice. The tumorigenic phenotype is strongly suppressed in cell hybrids formed between a benzo[*a*]pyrene-induced tumor cell line (BP6T) and normal SHE cells or early passage preneoplastic lines. At later passages, however, the ability of preneoplastic cell populations to suppress tumorigenicity becomes heterogeneous (*19*). A series of variants that differ more than 15- to 30-fold in their ability to suppress the tumorigenicity of BP6T cells (Table 1) have been subcloned from two independent preneoplastic lines (DES4 and 10W) (*23*). These subclones are designated $supB^+$ or $supB^-$ since BP6T cells represent only one of at least three different complementation groups for tumor suppression of hamster sarcomas (*24*). Interestingly, the $supB^-$ phenotype co-segregates with the ability to reversibly grow with high cloning efficiencies in soft agar supplemented with tumor cell-conditioned media or a combination of epidermal growth factor, insulin, and platelet-derived growth factor (*19,23*).

Identification of Genes Preferentially Expressed in Cells Retaining the Ability to Suppress Tumorigenicity

To isolate candidates for the BP6T tumor-suppressor gene function, we have used two approaches to identify genes that are preferentially expressed in $supB^+$ cells relative to $supB^-$ cells. The feasibility of these approaches is enhanced by the fact that the $supB^+$ and $supB^-$ cell lines are very closely related, having been subcloned from the same parental lines. In addition, we have used $supB^+$ and $supB^-$ variants derived from two independent parental cell lines, i.e., DES4 and 10W (*23*); this should increase the probability that the alterations we have detected are directly related to the $supB^+$ phenotype rather than simply fortuitous events.

Table 1. Suppression of BP6T cell tumorigenicity by $supB^+$ and $supB^-$ clonal variants.

Parental cells[a]	Tumor suppression ratio[b]	Fold suppression[c]	Tumor suppression phenotype
DES4	0.90	1.1	$supB^-$
DES4	1.05	1.0	$supB^-$
DES4	0.039	25	$supB^+$
DES4	0.031	32	$supB^+$
10W	0.95	1.0	$supB^-$
10W	1.11	0.9	$supB^-$
10W	1.07	0.9	$supB^-$
10W	0.037	27	$supB^+$
10W	0.066	15	$supB^+$

[a]DES4 (passage 60) and 10W (passage 17) cells were subcloned by one of two methods, i.e., colonies growing in agar supplemented with BP6T tumor cell-conditioned medium (conditionally transformed) or colonies growing in normal medium on plastic tissue culture dishes.

[b]The ratio of hybrid colonies formed with BP6T cells that grow in agar to total hybrid colonies formed on plastic.

[c]1/tumor suppression ratio. These data were modified from Koi et al. (*23*) with permission.

Initially, we examined the levels of more than 1000 [^{35}S]methionine-labeled proteins from total cell lysates of the $supB^+$ and $supB^-$ subclones using the Quest system (*25*) for quantitative two-dimensional gel electrophoresis. These studies were performed in collaboration with Michael Lambert and James Garrels (Cold Spring Harbor Laboratory). Given the sensitivity of this type of analysis, specific protein levels were very similar between the two cell types, as expected for these clonal derivatives. The only consistent alterations that were detected between each of the $supB^+$ and $supB^-$ cell lines were reductions of two high molecular weight tropomyosins in $supB^-$ cells (*26*). These tropomyosin isoforms were identified by comparisons with two-dimensional gel patterns of rat and human proteins as well as by immunoprecipitation with tropomyosin-specific antisera. Tropomyosin 1 and tropomyosin 2 levels were decreased 6-fold and 2-fold, respectively, in $supB^-$ cells relative to $supB^+$ cells. Analysis of silver-stained gels revealed similar reductions in the steady-state levels of these proteins in $supB^-$ cells. Likewise, comparable decreases in tropomyosin 1 mRNA were observed by Northern analysis with a rat tropomyosin cDNA probe in $supB^-$ cells.

Previous studies by several laboratories have reported decreased expression of high molecular weight tropomyosin isoforms in virally and chemically induced tumor cells relative to immortal precursor cells (*27–30*). However, our observations are the first example of altered tropomyosin expression in cells prior to neoplastic transformation. Stress fiber organization in $supB^+$ and $supB^-$ cells was also compared since tropomyosins are cytoskeletal proteins that are thought to stabilize actin microfilaments. Fluorescent microscopy of cells stained with rhodamine-phallodin demonstrated that the actin stress fibers of $supB^+$ cells were highly organized, while $supB^-$ cells consistently exhibited a diffuse, disorganized actin staining pattern common in tumor cells (*31*; J. Boyd, unpublished results). Studies using antisense techniques are underway to address a possible role for these cytoskeletal alterations in the $supB^+$ phenotype.

The second approach (Fig. 2) that we have adopted to identify genes that are preferentially expressed in $supB^+$ cells is the differential screening of cDNA libraries (*31*). A series of 31 $supB^+$ specific plaques were isolated by screening a DES4$supB^+$ cell lambda ZAP cDNA library sequentially with ^{32}P-labeled total cDNA probes prepared from DES4$supB^-$ and DES4$supB^+$ mRNA. For further analysis, cDNA inserts were excised from the lambda ZAP phage as pSK^- phagemid. These cDNA clones represented four different genes based on Northern analyses and cross-hybridization studies.

Northern analysis with two of these cDNAs as probes revealed transcripts of 5.1 kb and 4.5 kb, respectively, that are abundant in $supB^+$ cells but absent or barely detectable in normal SHE cells, $supB^-$ cells, and BP6T cells. DNA sequencing revealed that these cDNAs were

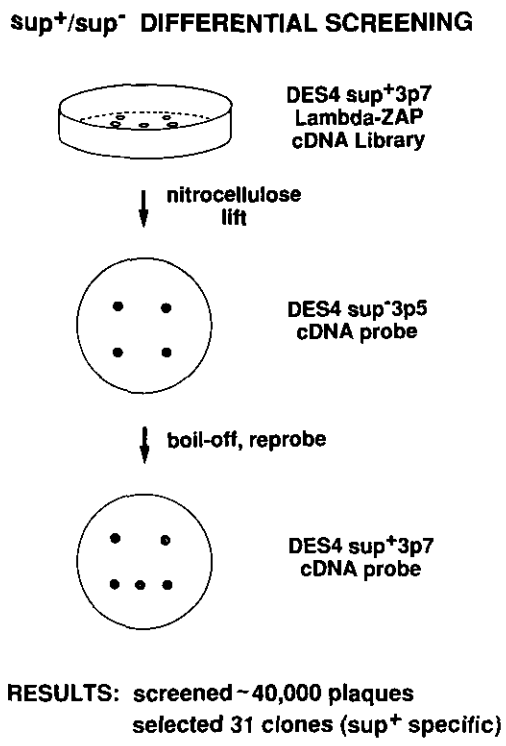

FIGURE 2. A cDNA library was constructed in lambda ZAP with mRNA from the DES4supB$^+$3 cell line. This library was sequentially screened with cDNA probes prepared from DES4supB$^-$3 and DES4supB$^+$3 mRNA. Thirty-one plaques that hybridized preferentially to the supB$^+$ were selected for further analysis.

the hamster homologs of the α1 chains of collagen type II and collagen type IX, which are normally expressed in chondrocytes (*37*). This suggests that the parental 10W and DES4 cell lines used in our studies are derived from a chondrocyte precursor or a chondrocyte-committed cell type. These cells must represent a minor subpopulation within primary SHE cell cultures since expression of these chondrocyte-specific collagens was barely detectable by Northern analysis of SHE cell RNA. It will be interesting to determine whether or not this cell type is a common target for carcinogen-induced immortalization of SHE cells.

The third cDNA detects a 2.4 kb transcript on Northern blots, which is expressed at high levels in SHE cells and DES4supB$^+$ lines but not in DES4supB$^-$ lines or BP6T cells. However, the levels of this mRNA do not consistently correlate with the supB$^+$ phenotype in the 10W subclones. DNA sequence analysis identified this cDNA as the hamster homolog of the H19 gene. Tilghman and co-workers (*38*) originally identified H19 by differential cDNA screening as a gene that was expressed coordinately with α-fetoprotein. This gene is developmentally regulated with expression of extremely high levels in many tissues during mouse embryogenesis, but its function remains unknown. Since no significant open reading frames are conserved among the mouse, human, and hamster homologs, the H19 gene may function at the RNA level (*39*).

Northern analysis with the remaining cDNA (clone 5) revealed a series of transcripts, with major species of 2.5 kb and 3.0 kb as well as several less abundant, larger mRNAs. These transcripts were observed in each of the supB$^+$ lines and normal SHE cells but were undetectable in the supB$^-$ lines and BP6T cells. DNA sequence analysis of over 2 kb at the 3′ end of this cDNA has failed to reveal any significant similarities with sequences currently in the GenBank or European Molecular Biology Laboratory databases.

In an attempt to understand the basis for the coordinate expression of these genes in supB$^+$ and supB$^-$ cells, nuclear run-on assays were performed for the collagen and H19 genes. These studies suggested that higher transcription rates were responsible for the increased steady-state levels of these transcripts in supB$^+$ cells. DNA sequence comparisons showed that a 9 bp element, TGT(T/C)TGCAG, that was previously identified in each of the mouse H19 enhancers was also present twice in the 5′ flanking region of the rat type II collagen gene (*36,40*). Preliminary gel retardation assays revealed the presence of factor(s) in nuclear extracts of supB$^+$ cells that bound to an oligonucleotide containing this conserved motif. Current studies using cDNA expression vector libraries are directed at the isolation of factors that bind to oligonucleotides containing this sequence element.

Conclusions

In this review, we have described two approaches that are directed toward the isolation of the supB$^+$ tumor-suppressor gene. Although the identity of this gene is still unknown, a series of genes whose expression is coordinately regulated with the supB$^+$ phenotype have been identified. If techniques such as differential cDNA screening and two-dimensional protein gel analysis are to succeed, it is essential that the cells being compared are as closely related as possible. The supB$^+$ and supB$^-$ cells that we have studied appear to fulfill this criteria. Furthermore, these techniques are based on the hypothesis that the expression of the supB$^+$ tumor-suppressor gene will be absent or greatly decreased in supB$^-$ cells. While this assumption is valid for several tumor-suppressor genes that have previously been cloned (*11,12*), genes like the P53 gene, which is functionally inactivated by point mutations (*41*), would be missed by these techniques.

The detection of a tumor-suppressor gene product by quantitative two-dimensional gel analysis is limited to moderately abundant proteins that are clearly resolved under the electrophoresis conditions employed. Likewise, isolation of the supB$^+$ gene by differential cDNA screening requires that this gene be expressed as a relatively abundant transcript (> 0.05% of the total mRNA) so that it is represented at a sufficient level in the ^{32}P-labeled probe to yield a detectable hybridization signal (*42*). However, subtractive hybridization techniques have been used to isolate considerably less abundant transcripts (*43,44*), and they provide an attractive

extension of the differential cDNA screening studies described in this report. Therefore, our current efforts are directed at the construction of a supB$^+$ cDNA library after several rounds of subtraction with supB$^-$ mRNA. Subtractive hybridization techniques using a mammalian expression vector may also be useful as an enrichment for supB$^+$ cDNAs with the potential to suppress the tumorigenicity of BP6T cells. Such a subtracted supB$^+$ library could be used in gene transfer assays like those described by Noda and co-workers (*16*) to screen for cDNAs with tumor-suppressing activity.

Of the genes identified whose expression is coordinately regulated with a tumor-suppressor function for BP6T cells, two (collagen type II and collagen IX) are differentiation markers, and another (H19) is a developmentally regulated gene. The loss of expression of these differentiation and developmental genes is consistent with the suggestion by Stanbridge and others (*5,8*) that tumor-suppressor genes may function by inducing differentiation *in vivo*. Several studies have implicated transcription factors in the control of differentiation, e.g., MyoD in the myogenic differentiation program (*44*). Since two copies of a 9 bp element, TGT(T/C)TGCAG, are conserved between the mouse H19 enhancers and the 5′ untranslated region of the rat type II collagen gene, we are currently investigating the existence of a potential transcription factor that binds this sequence and may control the coordinate expression of these genes with the supB$^+$ phenotype. Such a transcription factor would be an attractive candidate as a mediator of chondrocyte differentiation and the supB$^+$ phenotype, if not the supB$^+$ gene itself. This concept is especially intriguing in light of the recent report (*45*) of a role for the retinoblastoma gene product in transcriptional control of the c-*fos* promoter.

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